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Comparison of Performance Characteristics of Three Real-Time Reverse Transcription-PCR Test Systems for Detection and Quantification of Hepatitis C Virus▽

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We evaluated the performance characteristics of three real-time reverse transcription-PCR test systems for detection and quantification of hepatitis C virus (HCV) and performed a direct comparison of the systems on the same clinical specimens. Commercial HCV panels (genotype 1b) were used to evaluate linear range, sensitivity, and precision. The Roche COBAS TaqMan HCV test for research use only (RUO) with samples processed on the MagNA Pure LC instrument (Roche RUO-MPLC) and Abbott analyte-specific reagents (ASR) with QIAGEN sample processing (Abbott ASR-Q) showed a sensitivity of 1.0 $\log_{10}$ IU/ml with a linear dynamic range of 1.0 to 7.0 $\log_{10}$ IU/ml. The Roche ASR in combination with the High Pure system (Roche ASR-HP) showed a sensitivity of 1.4 $\log_{10}$ IU/ml with a linear dynamic range of 2.0 to 7.0 $\log_{10}$ IU/ml. All of the systems showed acceptable reproducibility, the Abbott ASR-Q being the most reproducible of the three systems. Seventy-six clinical specimens (50 with detectable levels of HCV RNA and various titers and genotypes) were tested, and results were compared to those of the COBAS Amplicor HCV Monitor v2.0. Good correlation was obtained for the Roche RUO-MPLC and Abbott ASR-Q ($R^2 = 0.84$ and $R^2 = 0.93$, respectively), with better agreement for the Abbott ASR-Q. However, correlation ($R^2 = 0.79$) and agreement were poor for Roche ASR-HP, with bias relative to concentration and genotype. Roche ASR-HP underestimated HCV RNA for genotypes 3 and 4 as much as 2.19 $\log_{10}$ IU/ml. Our study demonstrates that Roche RUO-MPLC and Abbott ASR-Q provided acceptable results and agreed sufficiently with the COBAS Amplicor HCV Monitor v2.0.

Hepatitis C virus (HCV) is still a major health care problem worldwide, including the United States. It is estimated that more than 170 million people worldwide are infected with HCV. HCV is one of the leading indications for liver transplantation in the United States (11). Prospective studies have shown that 60 to 85% of HCV-infected individuals develop chronic disease. Current recommendations for treatment are combination therapy with pegylated interferon and ribavirin (18). Treatment guidelines and monitoring the response to therapy rely heavily on viral load testing and genotype information. Genotyping should be performed before starting treatment to determine duration of treatment and dosage of ribavarin and also to provide prognostic information. The recommended durations of combination therapy are 24 weeks for genotypes 2 and 3 and 48 weeks for genotypes 1, 4, and 6 (18). Responses to treatment vary greatly, from approximately 40% for those infected with HCV genotypes 1 and 4 to about 80% for those infected with HCV genotypes 2 and 3.

Quantitative testing should be performed by methods with a wide enough dynamic range for accurate assessment of both pretreatment viral loads as well as an early virologic response, which is defined as a fall in the HCV RNA levels by at least 2 $\log_{10}$ units or to an undetectable level at week 12 of treatment. Currently, the best indicator of effective treatment is a sustained virologic response (SVR), defined by the absence of detectable HCV RNA in the serum when determined 6 months after the end of treatment. A sensitive method with a low limit of detection of 50 IU/ml or less should be used to assess SVR (18).

A variety of assays are commercially available to detect and quantify HCV RNA. They are based on three specific methodologies: PCR, transcription-mediated amplification (TMA) and the branched-DNA technique (4, 5, 15, 20). The most commonly used PCR-based assay for HCV quantification is the COBAS Amplicor HCV Monitor v2.0, with a lower limit of quantification of 600 IU/ml, which is inadequate to define an end-of-treatment response or SVR. Moreover, since this assay has an upper limit of quantification of 800,000 IU/ml, samples with baseline viral loads greater than the upper limit of quantification have to be diluted and retested to determine an early virologic response (5, 19, 27). The Versant HCV RNA v3.0 (Bayer HealthCare, Tarrytown, NY) uses branched-DNA technology for quantification and has a linear dynamic range of 4.1 $\log_{10}$ with a lower limit of quantification of 615 IU/ml, which is also inadequate for end-of-treatment assessments (5, 10, 21), making it necessary to use another method for evaluation of SVR at the end of treatment.

The advent of real-time reverse transcription-PCR (RT-PCR) combines high analytical sensitivity with wider dynamic range of quantification in a single platform, eliminating the

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need for separate quantitative and qualitative assay formats to meet the NIH guidelines (6, 12, 16, 18, 26). There are currently on the market three different products for HCV detection and quantification by real-time RT-PCR: the COBAS TaqMan HCV ASR, the COBAS TaqMan HCV test, and the HCV quantification ASR.

The COBAS TaqMan HCV test (Roche RUO; Roche Molecular Systems, Inc., Branchburg, NJ) is a real-time nucleic acid amplification assay for detection and quantification of HCV RNA in human serum or plasma. Roche markets this test as a research-use-only (RUO) test, and it is comprised of two products, the High Pure system viral nucleic acid kit for manual extraction of RNA (HP; Roche Molecular Systems) and the COBAS TaqMan HCV test kit, which can be purchased separately. Automated sample preparation is also suitable with the MagNA Pure LC instrument (MPLC; Roche Applied Science, Indianapolis, IN). The FDA considers RUO products to be products that are in the laboratory research phase of development with potential usefulness that can be adapted to clinical testing. The reagents in an RUO test are calibrated by the manufacturer and come with additional controls and instructions for use. Calculation of the HCV RNA titer is based upon an external standard curve in the presence of an internal control. This assay has been developed for use with the COBAS TaqMan 48 analyzer (CTM48; Roche Molecular Systems).

There are also on the market HCV analyte-specific reagents (ASRs) for HCV real-time RT-PCR, the ASR version of the TaqMan HCV test (Roche ASR; Roche Molecular Systems) and the HCV quantification ASR (Abbott ASR; Abbott Diagnostics, Abbott Park, IL). The FDA considers ASRs as building blocks of laboratory-developed tests used exclusively by the laboratory that purchases the product. Under Clinical Laboratory Improvement Amendments regulations, the clinical laboratories are required to develop and maintain the analytical performance characteristics of the test in which the ASR is used and to report the test results with a standard disclaimer.

These reagents and tests potentially offer many advantages over current commercially available ones, including expanded dynamic range, improved sensitivity and increased automation, high throughput, and decreased turnaround time. Currently, there are a limited number of published peer-reviewed studies evaluating the performance characteristics of these reagents and platforms (1, 3, 6, 8). Moreover, there are no published data comparing these reagents and platforms using the same clinical specimens.

Three different test systems were evaluated in this study, using HCV RNA reference materials in which certain extraction methods were combined with certain real-time RT-PCR reagents: (i) the COBAS TaqMan HCV ASR with manual sample preparation with the Roche High Pure system (Roche ASR-HP), (ii) the COBAS TaqMan HCV test with automated sample preparation on the MPLC instrument (Roche RUO-MPLC), and (iii) the HCV quantification ASR with manual sample preparation with the QIAamp viral RNA Mini kit (Abbott ASR-Q).

We chose to combine the Roche High Pure system with the TaqMan HCV ASR to evaluate the Roche real-time reagents along with a Roche manual sample processing method that might be useful for a clinical laboratory to replace the COBAS Monitor in routine diagnostic testing. We combined the Roche MagNA Pure sample preparation with the TaqMan HCV RUO because implementation of automated sample processing might be beneficial for clinical laboratories with increasing numbers of samples. The TaqMan HCV RUO became available at the time we performed the study and does not require external calibration, which allows for standardization and fewer requirements by the laboratories. We also combined the QIAGEN viral RNA Mini kit manual extraction method with the Abbott ASR based on recommendations provided by consultants that had evaluated this system in their laboratories with accurate results and also based on our experience using this extraction method for other clinical testing.

After performing the evaluation of these systems, we analyzed clinical specimens that were previously tested in our laboratory by the COBAS Amplicor HCV Monitor v2.0 test. We compared the HCV RNA results of the real-time RT-PCR systems with the COBAS Monitor results for correlation and agreement and to evaluate performance for quantification of different genotypes.

MATERIALS AND METHODS

HCV reference material. Two commercially available panels of HCV genotype 1b standards calibrated against the WHO International Standard for HCV RNA were used for linearity and sensitivity studies: (i) the OptiQuant HCV RNA (Acrometrix Corp., Benicia, CA) with seven members at 5,000,000, 500,000, 50,000, 5,000, 500, 50, and 0 IU/ml and associated dilutions (25, 10, and 5 IU/ml); and (ii) the HCV linearity panel (PHW804; BBI Diagnostics, MA) with six members at 10,000,000, 1,000,000, 250,000, 110,000, 11,000 and 1,600 IU/ml and three associated dilutions down to 1.6 IU/ml. In addition, three different HCV RNA reference materials at 910,000, 5,000, and 100 IU/ml were used for evaluation of precision (Accurun reference no. 405, 305, and 306, respectively) (Boston Biomedica, Inc., Boston, MA). The HCV RNA reference materials of 5,000 and 100 IU/ml were also used as positive controls in every run for the Roche ASR. Calibration of the Roche ASR was performed with reference materials of 910,000, 5,000 and 1,000 IU/ml, respectively. Dilutions were prepared by using residual HCV-negative human plasma from a single plasmapheresis patient that was stored at −70°C until analysis. The HCV RNA values in IU/ml obtained by the manufacturer using the COBAS Amplicor HCV Monitor v2.0 test were used as expected values to evaluate the performance characteristics of the systems.

Clinical specimens. To evaluate quantitative correlation and performance related to different genotypes, a total of 76 residual plasma specimens (50 specimens with detectable levels of various HCV RNA titers and genotypes, except genotype 5, and 26 undetectable specimens) submitted to our laboratory between January 2002 and September 2003 for routine quantitative HCV RNA testing were retrospectively selected for this study. The group of well-characterized plasma specimens had HCV RNA titers ranging from 3.97 to 7.09 $\log_{10}$ IU/ml, as determined by COBAS Amplicor HCV Monitor test v2.0 (Roche Diagnostics Corporation, Indianapolis, IN) performed according to the manufacturer's instructions. Specimens with concentrations greater than 500,000 IU/ml (5.7 $\log_{10}$) were diluted 1:100 in HCV-negative plasma and retested (13, 14, 17). These specimens included 26 HCV genotype 1, 8 HCV genotype 2, 6 HCV genotype 3, 7 genotype 4, and 3 genotype 6, as determined by Innolipa assay (Versant HCV genotype assay; Bayer Diagnostics, Tarrytown, NY) performed according to the manufacturer's instructions (13, 14, 17). Specimens were stored at −70°C from 4 to 84 weeks following the COBAS Monitor testing. The study was reviewed and approved by the Medical College of Virginia Institutional Review Board.

Real-time RT-PCR test systems. (i) Roche RUO-MPLC system. HCV RNA was isolated from a 500-μl aliquot of each of the reference materials, controls, and clinical specimens using the automated MPLC instrument with software version 3.03 and a Roche total nucleic acid isolation kit (large volume; Roche Applied Science, Indianapolis, IN). The manufacturer's protocol was followed with the following modifications. The HCV internal quantification standard (QS) was added directly to the lysis/binding buffer in order to achieve full process control. For processing of 24 samples, 104 μl of the internal QS was added to 34.4 ml of MagNA Pure lysis/binding buffer and gently mixed prior to being

dispensed into the appropriate MPLC reagent reservoir. After completion of several washing steps, purified nucleic acids were eluted in 75 μl of elution buffer at elevated temperature.

The master mix was activated by the addition of 170 μl of 50 mM manganese acetate and used within 60 min of preparation. Fifty microliters each of samples from reference materials, controls, and clinical specimens was combined with 50 μl of master mix and processed for amplification and detection using the COBAS TaqMan 48 analyzer according to the manufacturer's instructions with a single lot of reagent throughout the study. The COBAS TaqMan 48 instrument with Amplilink software v3.0.1 (Roche Molecular Systems) automatically determines the HCV titer for each sample and control based upon an external standard curve with the calibration coefficients that are specific for each lot of reagents.

(ii) Abbott ASR-Q system. HCV RNA was extracted from 220-μl aliquots each of reference material and controls using the QIAamp viral RNA Mini kit (QIAGEN, Valencia, CA). The extraction was done per the manufacturer's instructions with the following modifications. First, 15.5 μl of HCV RNA ASR (Abbott Diagnostics, Abbott Park, IL) with an initial concentration of 4.38×10^6 IU/ml was added to the lysis buffer containing carrier RNA. The extracted RNA was then eluted in 80 μl of elution buffer. The Abbott internal QS (HCV RNA ASR) is an armored RNA that is added to the lysis buffer to prevent degradation. HCV RNA ASR contains RNA sequences complementary to the primers used to amplify HCV and an intervening scrambled sequence that is used to detect the HCV RNA ASR during the real-time RT-PCR. HCV RNA ASR is used for quantification of HCV RNA and to correct for the presence of reaction inhibitors and other variables that can affect the efficiency of real-time PCR.

Fifty microliters each of samples from reference materials and controls was loaded into a 96-well optical reaction plate containing 50 μl of reaction mixture in each well, and the real-time RT-PCR was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the following thermal cycling parameters: a first stage of activation of AmpErase uracil-*N*-glycosylase (4 min at 50°C), followed by an RT (30 min at 60°C) and 50 PCR cycles of 91°C for 15 s and 60°C for 1 min. Sequence Quantification Software v2.0 (Abbott Diagnostics, Abbott Park, IL) was used to calculate the HCV RNA concentration in the samples by comparing the signals generated by the target and the internal calibrator.

Clinical specimens were analyzed next, following the aforementioned optimized protocol to assess correlation.

(iii) Roche ASR-HP system. HCV RNA was isolated from 500 μl each of the reference material and controls using the manual High Pure system viral nucleic acid kit procedure (Roche Applied Science, Indianapolis, IN). Sample preparation was done according to the manufacturer's instructions, adding the appropriate volume of HCV internal QS RNA to each specimen so that the final concentration of the QS after HCV RNA isolation was approximately 1,100 copies per reaction tube. Nucleic acids were eluted from glass fiber particles with 75 μl of elution buffer.

Working master mix was prepared by combining manganese (170 μl) and ASR master mix reagents (1.4 ml). The working master mix (50 μl) was added to each K-tube in the K-carrier, followed by 50 μl of each test sample. Samples were processed for amplification and detection on the COBAS TaqMan 48 analyzer using the following thermal cycling parameters: two precycles of 5 min at 50°C and 30 min at 59°C and 25 cycles of 15 s at 95°C and 25 s at 58°C followed by 25 cycles of 15 s at 91°C and 25 s at 58°C and a postcycle hold at 40°C. A cycle delay of 25 s was set up during the annealing steps. The TaqMan HCV ASR requires user calibration with each new lot of reagents. For this study, one lot of Roche ASR reagents was used and calibration was performed using commercially available reference materials. The HCV QS was introduced during sample preparation into reference materials with known concentrations of HCV RNA genotype 1b (910,000, 5,000, and 1,000 IU/ml previously assigned by the commercial vendor against the WHO 1st International Standard for HCV RNA). Six replicates of each reference material were then processed to isolate HCV and HCV QS RNA as mentioned above. The resulting samples were amplified and detected in the COBAS TaqMan analyzer. The threshold values for both HCV and HCV QS obtained for each calibrator were saved and exported as text files. The text files were imported into ASR external calibration software (Roche Molecular Systems, Inc., version 2.1) to derive three coefficients. The entire process was repeated three times, and calibration coefficients were calculated for each run. The average of each calibration coefficient defined the calibration curve for the ASR-specific lot, and they were used in the calculation of sample HCV RNA concentrations.

Clinical specimens were next tested for correlation study following the aforementioned protocol with the same TaqMan HCV ASR lot number and quantified using those calibration coefficients.

Quantitative correlation and genotype performance. The data obtained from the analysis of the 76 clinical specimens by the real-time RT-PCR systems were compared with previous results of HCV viral load by COBAS Amplicor HCV Monitor v2.0 assay. Correlation, agreement, and performance related to genotypic differences between the two methods were assessed. The difference between the $\log_{10}$ HCV RNA IU/ml results obtained from the real-time RT-PCR and the COBAS Monitor were plotted against their average in $\log_{10}$ HCV RNA IU/ml to determine the agreement between the two methods. Since, the true value was not determined by a reference method, the average of the two measurements was the best alternative. The limits of agreement for the real-time RT-PCR HCV systems were also calculated. Performance with relation to genotype for the Roche ASR-HP was evaluated, and the quantification differences between both assays (Roche ASR-HP versus COBAS Monitor) were plotted against their average to estimate the genotype bias.

Statistical analysis. HCV concentrations were $\log_{10}$ transformed for analysis. The linear range was examined by plotting the data and comparing them to a line of equality. Correlation coefficients and linear regression analysis were done in scatter plots for log-transformed HCV RNA levels using Microsoft Excel (Microsoft Office 2000; Microsoft Corp., Redmond, WA). Agreement between the COBAS Monitor and the real-time RT-PCR systems was determined by Bland-Altman plot (2). Potential differences in target amplification efficiency were examined by comparing the slopes of the linear regression lines.

RESULTS

Linearity of the real-time RT-PCR systems. The HCV RNA reference materials of genotype 1b were extracted and run in triplicates on 3 different days by Roche RUO-MPLC, Abbott ASR-Q, and Roche ASR-HP to establish the linear range of the systems. Data detailing the results are presented in Fig. 1. Results were plotted using the log-transformed values of the nominal HCV RNA input concentrations and the log-transformed values of the HCV RNA measured concentrations and analyzed using linear regression.

Roche RUO-MPLC. Regression analysis showed a linear correlation between the Roche RUO-MPLC and the expected HCV RNA values ($R^2 = 0.994$) (Fig. 1A). The slope of the curve was 0.896, but it is interesting to note that for values ≥ 2.70 $\log_{10}$ IU/ml, the mean measured values were lower than nominal values by about 0.23 $\log_{10}$ IU/ml and the mean measured values were 0.23 $\log_{10}$ IU/ml higher than the nominal values at concentrations <2.70 $\log_{10}$ IU/ml. The Roche RUO-MPLC was linear in the full range tested (1.0 to 7.0 $\log_{10}$ IU/ml), with high reproducibility and a mean coefficient of variation (CV) of 5.34% in $\log_{10}$ IU/ml. These concentrations met the criterion of acceptable precision and trueness (standard deviation [SD], < 0.25) used to define the lower and higher limits of quantification for this particular assay.

Abbott ASR-Q. Linear correlation between the Abbott ASR-Q and the expected HCV RNA values was obtained ($R^2 = 0.995$) with a slope close to 1.00 (Fig. 1B). The HCV RNA measured concentrations were consistently lower than the nominal concentrations of 0.26 $\log_{10}$ IU/ml as an average. The assay was linear between 1.0 and at least 7.0 $\log_{10}$ IU/ml. The Abbott ASR-Q lower and higher limits of quantification were established at those concentrations since they met the criterion of acceptable precision and trueness (SD, <0.25).

Roche ASR-HP. The Roche ASR-HP showed a linear correlation between nominal and measured concentrations ($R^2 = 0.985$) (Fig. 1C). The slope of the linear regression line was close to 1.0, and HCV RNA results were lower than the nominal value of 0.02 $\log_{10}$ IU/ml as an average. The assay was linear and very reproducible between 2.0 and at least 7.0 $\log_{10}$ IU/ml, with an average CV of 2.99% in $\log_{10}$ IU/ml. The lower

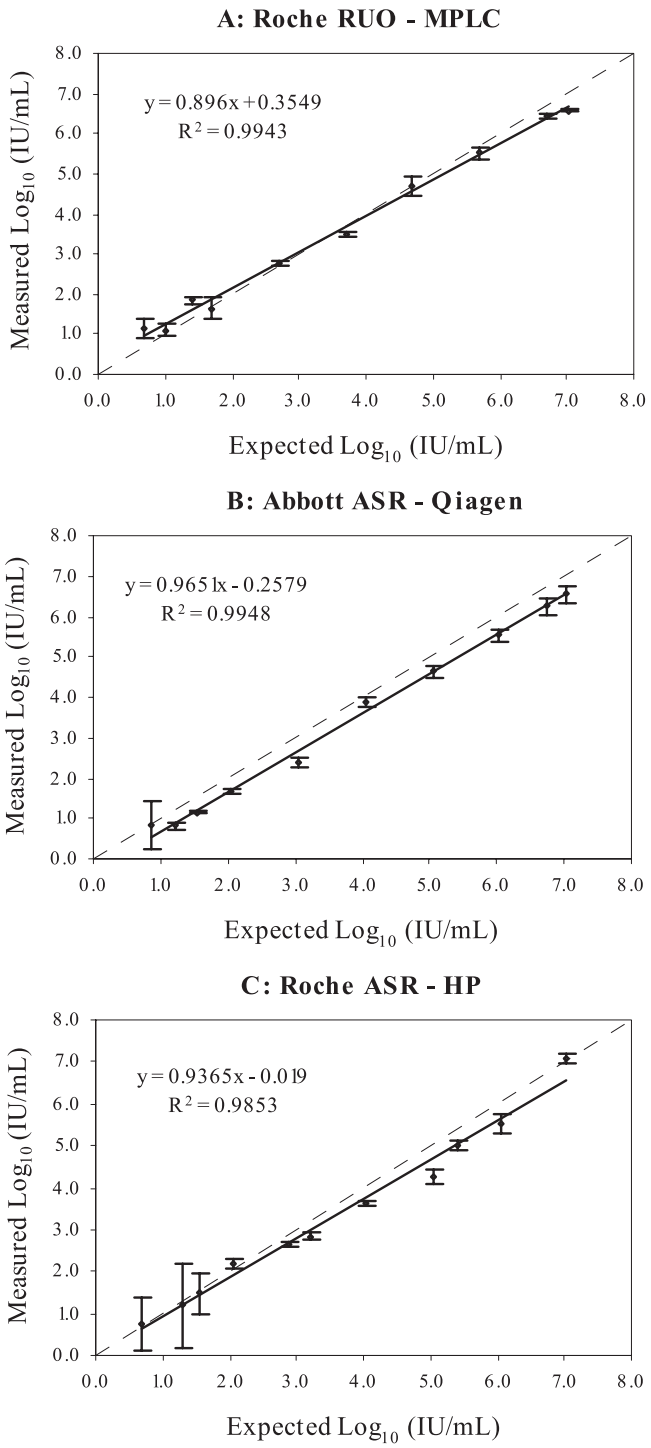

FIG. 1. Linearity of the real time RT-PCR systems. Linear range was determined by regression analysis using the expected HCV RNA concentrations and the corresponding test results. Each point represents the mean $\log_{10}$ HCV IU/ml of three replicates tested on 3 different days. The dashed line represents equality.

and higher limits of quantification were determined at those values, respectively (SD, <0.25).

Precision. Reproducibility was evaluated by using three different levels of reference material of genotype 1b with 910,000, 5,000, and 100 IU/ml HCV RNA levels that were run in duplicate in six runs performed on 6 different days. The three systems showed acceptable reproducibility, with interassay CV ranging from 2.46% to 9.29% in $\log_{10}$ IU/ml for the higher level and the lower level of reference materials, respectively. The Abbott ASR-Q was the most reproducible of the three systems.

Analytical sensitivity. Six serial dilutions (500, 100, 50, 25, 10, and 5 IU/ml) of a reference material with 5,000 HCV RNA IU/ml (NAP; Acrometrix, Benicia, CA) were run in duplicate on 3 different days to determine the sensitivity of the real-time RT-PCR systems. The Roche RUO-MPLC and Abbott ASR-Q systems were able to detect all six replicates with 10 HCV RNA IU/ml (1.0 $\log_{10}$ IU/ml), and the Roche ASR-HP system was able to detect all six replicates with 25 HCV RNA IU/ml (1.4 $\log_{10}$ IU/ml).

Quantitative correlation of the real-time RT-PCR systems and the COBAS Monitor. Results obtained from the analysis of the 76 clinical specimens by real-time RT-PCR systems were compared with previous results obtained from COBAS Amplicor HCV Monitor v2.0 assay. The clinical sensitivity and specificity of the TaqMan systems were 100%, respectively; 26 of 76 clinical specimens with undetectable results by COBAS Monitor were also undetectable by the TaqMan systems, and 50 of 76 clinical specimens with detectable results by COBAS Monitor were also detectable by the TaqMan systems. Quantitative correlations between the COBAS Monitor and the real-time RT-PCR systems were established in the 50 clinical specimens with detectable HCV RNA values by both methods, and the results are plotted in Fig. 2. Mean viral load values, SD of the mean, and quantification differences between the real-time RT-PCR systems and COBAS Monitor were also calculated by genotype and are represented in Table 1.

Roche RUO-MPLC with COBAS Monitor. Comparison of Roche RUO-MPLC with COBAS Monitor showed a good correlation by linear regression analyses ($R^2 = 0.840$; $P < 0.0001$) with a slope close to 1 (Fig. 2). The Roche RUO-MPLC results were consistently lower than the COBAS Monitor results. Genotype 3 samples showed a slightly higher degree of underestimation compared to genotypes 1, 2, and 4 (Table 1).

Abbott ASR-Q with COBAS Monitor. Comparison of Abbott ASR-Q with COBAS Monitor showed a strong correlation by linear regression analyses ($R^2 = 0.925$; $P < 0.0001$) independent of genotype. The slope of the curve was close to 1, with HCV RNA results being slightly lower than COBAS Monitor results for most of the samples (Fig. 2 and Table 1).

Roche ASR-HP with COBAS Monitor. Poor correlation was obtained when comparing Roche ASR-HP results with those from the COBAS Monitor ($R^2 = 0.788$; $P < 0.0001$) (Fig. 2). Characteristics of the regression line included a slope of 1.25 and a y intercept of -1.73. Data analysis demonstrated that Roche ASR-HP results depended on HCV RNA concentration and genotype (Table 1); hence, a detailed analysis for each genotype was performed for this assay.

To determine whether genotype-specific quantification bias occurs in the Roche ASR-HP, the 50 positive HCV RNA samples were analyzed by genotype and compared to the COBAS Monitor results. Good correlation was obtained for HCV genotype 1 ($R^2 = 0.940$; $P < 0.0001$). However, the slope

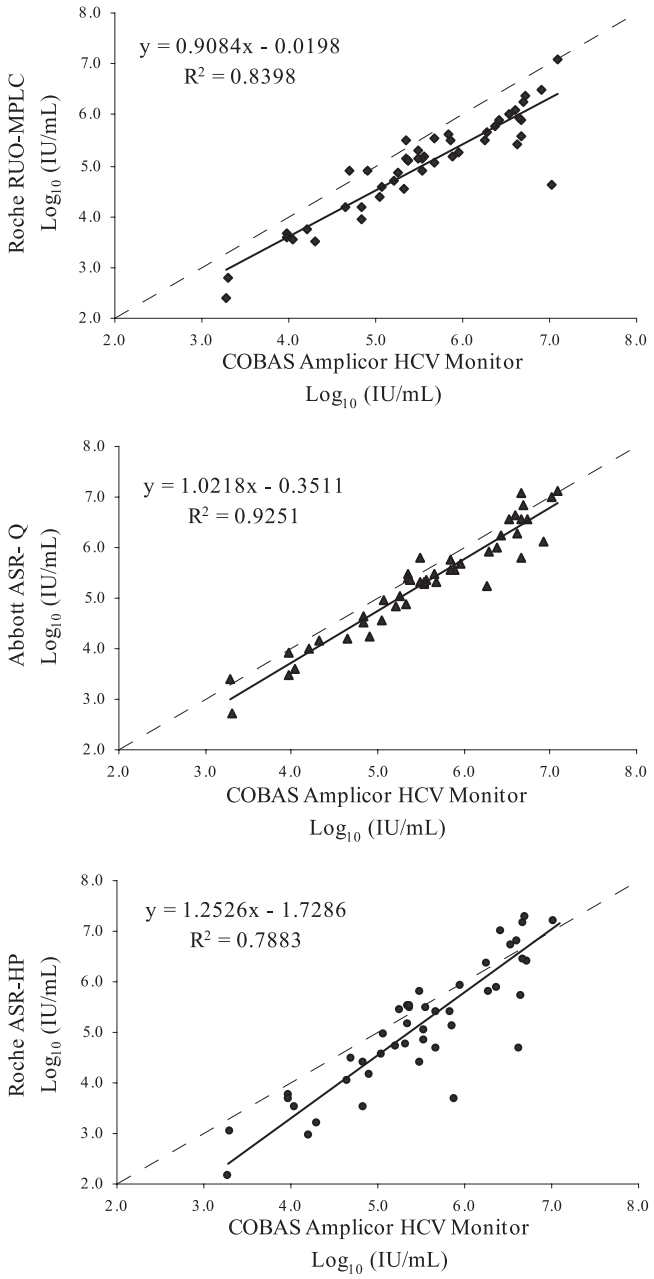

FIG. 2. Correlation of results for the real-time RT-PCR systems and COBAS Amplicor HCV Monitor v2.0. The dashed line indicates the line of equality.

of the curve was 1.36, with an overestimation of the HCV RNA concentrations by the Roche ASR-HP for samples with COBAS Monitor results higher than 6.4 $\log_{10}$ IU/ml and underestimation of the HCV RNA concentrations for samples with lower than 5.30 $\log_{10}$ IU/ml by COBAS Monitor. Four of 26 samples had HCV RNA results lower than 0.5 $\log_{10}$ IU/ml compared to COBAS Monitor. Regression analysis for genotype 2 ($R^2 = 0.802$; $P < 0.005$) and genotype 4 ($R^2 = 0.700$; $P < 0.05$) showed poor correlation. Two of the eight genotype 2 samples and four of the seven genotype 4 samples had HCV RNA results of about 1.0 $\log_{10}$ IU/ml lower than COBAS Monitor results. There was no correlation for genotype 3 ($R^2 = 0.495$; $P < 0.12$), and four of six samples showed an underestimation by 1.24 to 2.19 $\log_{10}$ IU/ml using the Roche ASR-HP system. Regression analysis for genotype 6 was not performed due to the small number of samples. Quantification differences between the Roche ASR-HP and the COBAS Monitor were also plotted against their average to estimate the genotype bias (Fig. 3).

Agreement between the real-time RT-PCR systems and the COBAS Monitor. A Bland and Altman plot was used to determine the agreement between the Roche RUO-MPLC, Abbott ASR-Q, and Roche ASR-HP systems and the COBAS Monitor. Using this method, the differences between the real-time RT-PCR systems and the COBAS Monitor were plotted against the averages of the two techniques.

Roche RUO-MPLC with COBAS Monitor. The Roche RUO-MPLC results were consistently lower than those of the COBAS Monitor. The mean difference between values (Roche RUO MPLC values − COBAS Monitor values) was −0.49 $\log_{10} \pm 0.22$, with limits of agreement (mean difference ± 2 SD) of −0.05 and −0.93 $\log_{10}$ IU/ml. No bias in relation to concentration was observed (Fig. 4A).

Abbott ASR-Q with COBAS Monitor. Results from the Abbott ASR-Q were in good agreement with the COBAS Monitor. The mean difference between the real-time RT-PCR values and the COBAS Monitor values was −0.20 $\log_{10} \pm 0.24$, with limits of agreement of 0.29 and −0.69 $\log_{10}$ IU/ml, and more than 95% of the differences fell within these limits. In concordance with Roche RUO-MPLC, no bias in relation to concentration was observed (Fig. 4B).

Roche ASR-HP with COBAS Monitor. The agreement between Roche ASR-HP and COBAS Monitor was poor, with a mean difference of −0.33 $\log_{10} \pm 0.68$ between the results (Roche ASR-HP and COBAS Monitor). The differences tend to be negative for samples with viral load values of <5.0 $\log_{10}$ IU/ml, equally distributed around the mean difference for samples with viral load values between 5.0 and 6.4 $\log_{10}$ IU/ml, and positive for samples with viral load values of >6.4 $\log_{10}$ IU/ml (Fig. 4C).

As previously described, a bias related to genotype was found. Agreement plots between the Roche ASR-HP and the COBAS Monitor for genotypes 1 to 4 are shown in Fig. 3.

DISCUSSION

HCV RNA quantification is an essential tool for management of acute and chronic hepatitis C. Thus, the method used to quantify HCV RNA should be reliable across all the different HCV genotypes and have a high sensitivity and a wide dynamic range.

Our evaluation of the performance characteristics of RT-PCR systems clearly demonstrated the advantages of using real-time RT-PCR in monitoring HCV patients. The Roche RUO-MPLC and Abbott ASR-Q were demonstrated to be accurate and reliable systems capable of providing quantitative results in a single test that meets the current NIH guidelines for treatment decisions and therapy monitoring.

Our analytical data suggest that the Roche RUO-MPLC and Abbott ASR-Q systems are very sensitive, detecting 100% of the replicates with 1.0 $\log_{10}$ HCV RNA IU/ml. Even though

TABLE 1. Quantitative values by HCV genotype for real-time RT-PCR systems in 50 clinical specimens

HCV genotype	Genotype quantification in mean $\log_{10}$ IU/ml (SD)				Quantification difference (RT systems − COBAS Amplicor)[a]		
	COBAS Amplicor	Roche RUO-MPLC	Abbott ASR-Q	Roche ASR-HP	Roche RUO-MPLC	Abbott ASR-Q	Roche ASR-HP
1	5.43 (1.08)	5.03 (1.00)	5.23 (1.23)	5.35 (1.51)	−0.4	−0.2	−0.08
2	5.80 (1.05)	5.35 (1.02)	5.64 (0.88)	5.61 (1.47)	−0.45	−0.16	−0.19
3	5.23 (1.05)	4.62 (0.89)	5.01 (0.99)	3.99 (0.89)	−0.61	−0.22	−1.24
4	5.69 (0.36)	5.22 (0.38)	5.4 (0.34)	5.02 (0.50)	−0.47	−0.29	−0.67

[a] Differences in quantification between the real-time RT-PCR systems and COBAS Amplicor Monitor show some trends in the performance of the systems, especially when broken down by genotype.

the Abbott ASR-Q system uses roughly half the starting sample volume, it was also able to detect 1.0 $\log_{10}$ HCV RNA IU/ml, similar to the Roche RUO-MPLC. In addition, they remained linear to at least 7.0 $\log_{10}$ HCV RNA IU/ml. Our study was limited to the analysis of reference material ranging from 1.1×10^7 IU/ml to undetectable and clinical samples with a higher titer of 1.23×10^7 IU/ml. We did not evaluate the higher reportable range of the COBAS TaqMan HCV test (2×10^8 IU/ml) as defined by the assay's manufacturer. The Roche RUO-MPLC results obtained from our study were consistent with those recently reported by Germer et al. with respect to the limit of detection and linear range (8). The limit of detection and linear range of the Roche RUO-MPLC and Abbott ASR-Q in our study were also in concordance with a recent study by Caliendo et al. (3), even though the authors used the QIAGEN MiniElute system for nucleic acid extraction. Both systems provided sensitivity equivalent to that of the commercially available HCV RNA qualitative assays (Amplicor HCV; Roche Diagnostics, Indianapolis, IN; and TMA-based Versant; Bayer HealthCare, Berkley, CA) and a higher dynamic range than the quantitative available assays (10). The combinations of high analytical sensitivity with a wider dynamic range of quantification in a single platform eliminate the need for multiple testing. The increased sensitivity of these systems could be important in the management of HCV-infected patients, since the increased sensitivity of transcription-mediated amplification has been shown to improve the detection of extremely low levels of HCV RNA in end-of-treatment specimens and improve the prediction of treatment failure or virologic relapse in patients receiving anti-HCV therapy (9, 24). The performance of the Roche ASR-HP with respect to the reference materials with genotype 1b showed lower sensi-

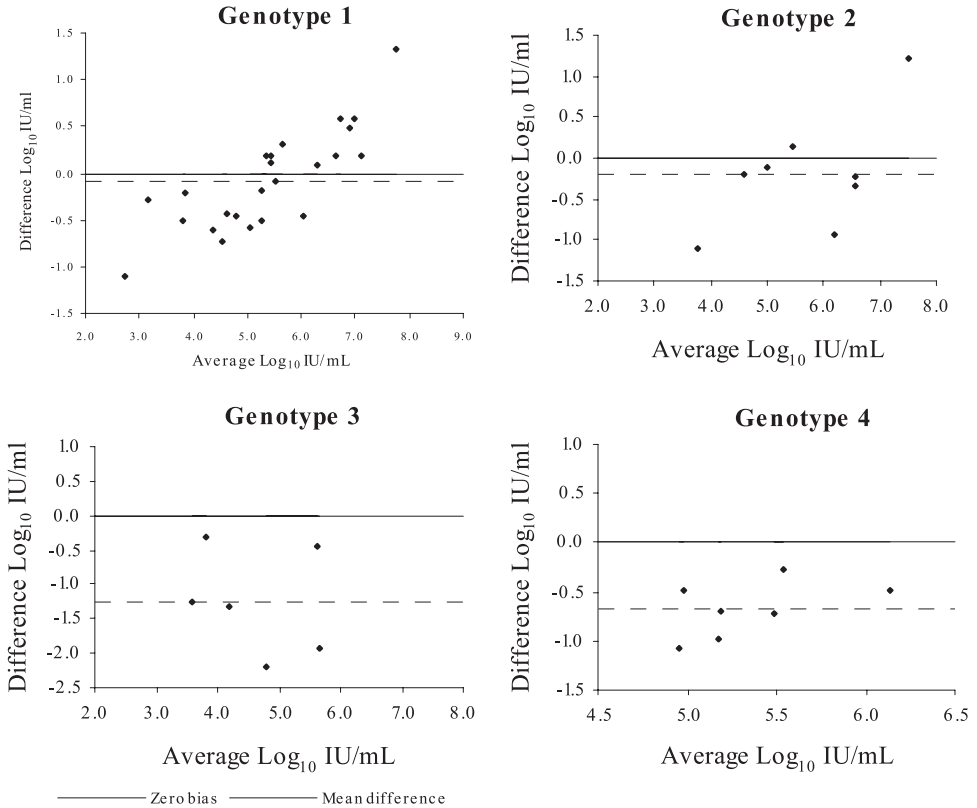

FIG. 3. Differences in quantification between Roche ASR-HP and COBAS Monitor in $\log_{10}$ HCV RNA IU/ml for genotypes 1 to 4.

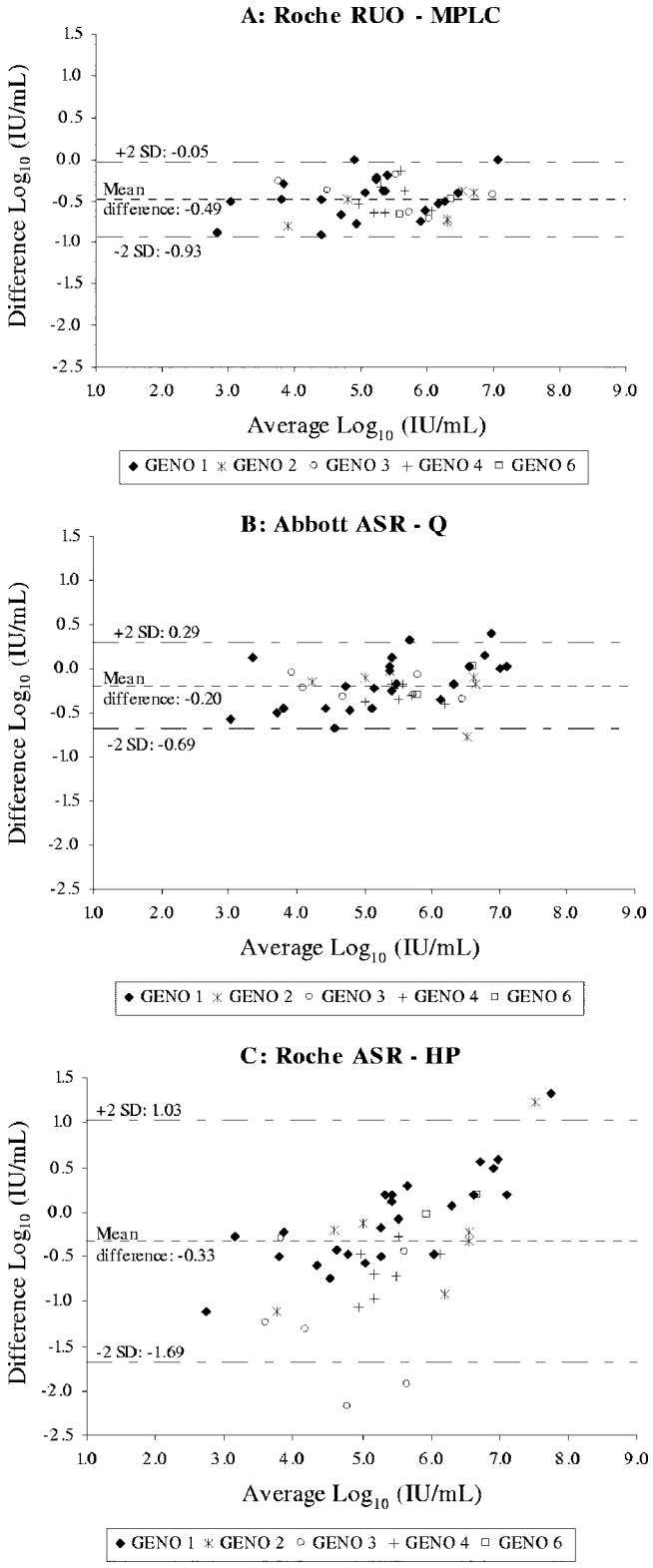

FIG. 4. Differences in $\log_{10}$ HCV RNA between the real-time RT-PCR systems and COBAS Monitor plotted against the averages of the two techniques. GENO, genotype.

tivity (1.4 $\log_{10}$ IU/ml) than the Roche RUO-MPLC and Abbott ASR-Q, with a linear dynamic range of 2.0 to 7.0 $\log_{10}$ HCV RNA IU/ml.

The Abbott ASR-Q was the most reproducible of the three systems tested. The Roche RUO-MPLC and the Roche ASR-HP were also quite reproducible for reference materials with HCV RNA values greater than 2.0 $\log_{10}$ IU/ml.

In dynamic range experiments using reference material of HCV genotype 1b, all three systems gave viral load values within 0.5 $\log_{10}$ IU/ml for all concentrations in the respective linear range of the systems. This level of agreement was remarkable, given that the systems had different extraction methods, cycling parameters, and instrumentations. In contrast, greater variability in quantification between the different systems was observed when clinical specimens were tested for correlation and performance with respect to different genotypes. The Roche RUO-MPLC and Abbott ASR-Q results from clinical specimens were in good correlation with those of COBAS Amplicor HCV Monitor v2.0. Results were lower than those of the COBAS Monitor for most of the samples tested, and no bias in relation to concentration was observed. There were no substantial differences in quantification among the various HCV genotypes, except for genotype 3 samples that were slightly underestimated by Roche RUO-MPLC. The average differences in quantification were less than 0.5 $\log_{10}$ IU/ml (within threefold), which are generally considered acceptable when comparing different methods for HCV viral load measurement (25), with a better level of agreement for the Abbott ASR-Q. It was recently noted that HCV Monitor v2.0 overestimated an international standard concentration by approximately 0.49 $\log_{10}$ IU/ml (22). The authors attributed the overestimation to an erroneous assignment of the number of IU/ml to the HCV RNA concentration of the QS. However, there is no "gold standard" quantitative assay for these types of studies and, for the purposes of this analysis, trends in viral load were more important than absolute quantification.

Comparison of Roche ASR-HP system with the COBAS Monitor in 50 clinical specimens with various HCV RNA titers and genotypes showed poor correlation and poor agreement between the two methods with bias relative to concentration and genotype. A significant underestimation of the HCV RNA levels for some samples with genotype 2 and most of the samples with genotypes 3 and 4 was observed. These results are consistent with those described by Sarrazin et al. and Gelderblom et al. (7, 23) using the High Pure system in combination with the COBAS TaqMan HCV RUO test. In contrast, Forman and Valsamakis and Barbeau et al. (1, 6) observed similar amplification efficiencies for genotypes 1 to 4 when QIAGEN manual and automated extraction methods and automated extraction on the MPLC instrument, respectively, were used in combination with the Roche ASR. According to Roche, the COBAS TaqMan ASR and RUO tests are equipped with the same primers and probes; therefore, genotype differences may be caused by components of the High Pure system (for example, buffer solutions) that might influence the unfolding of the HCV RNA, as was recently described by Gelderblom et al. (7). Although certain parts of the 5′ untranslated region (5′UTR) are highly conserved among genotypes, other parts of the 5′UTR and flanking regions are not. The tertiary and quaternary structures of the HCV RNA may differ among genotypes,

and sequence differences outside the 5′UTR may influence its folding and unfolding. Incomplete unfolding may (i) comprise binding of HCV RNA to glass fiber and cause loss of HCV RNA during the HP extraction and/or (ii) reduce binding of the primers and probe to the highly conserved 5′UTR of the HCV genome, both resulting in underestimation of HCV RNA in the specimen. Further studies are needed to assess the reasons for underestimation of some but not all of the samples with genotypes 2, 3, and 4.

In summary, this study describes the performance of three real-time RT-PCR systems and compares it with results obtained from the COBAS Monitor assay. Although the Abbott ASR-Q system performed slightly better than the Roche RUO-MPLC, especially in quantification of HCV genotype 3, both systems performed very well and agreed sufficiently with the established COBAS Amplicor HCV Monitor v2.0.

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